

RNA Interference in Functional Genomics and Medicine

RNA interference (RNAi) is the sequence-specific gene silencing induced by double-stranded RNA (dsRNA). Being a highly specific and efficient knockdown technique, RNAi not only provides a powerful tool for functional genomics but also holds a promise for gene therapy. The key player in RNAi is small RNA (~22-nt) termed siRNA. Small RNAs are involved not only in RNAi but also in basic cellular processes, such as developmental control and heterochromatin formation. The interesting biology as well as the remarkable technical value has been drawing widespread attention to this exciting new field.

Key Words : RNA Interference (RNAi); RNA, Small interfering (siRNA); MicroRNAs (miRNA); Small hairpin RNA (shRNA); mRNA degradation; Translation; Functional genomics; Gene therapy

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INTRODUCTION

The RNA interference (RNAi) pathway was originally recognized in *Caenorhabditis elegans* as a response to double-stranded RNA (dsRNA) leading to sequence-specific gene silencing (1). It soon turned out that RNAi is not restricted to nematode and can be induced in *Drosophila* (2), *Trypanosoma* (3), and vertebrates (4). Similar phenomena had been observed in plants and fungi, where introduction of exogenous transgenes silenced expression of the endogenous loci (5). These phenomena were called co-suppression (also termed post-transcriptional gene silencing, PTGS) and quelling, respectively. Co-suppression was shown later to be mediated by dsRNA as a guide molecule, establishing a mechanistic link to RNAi. This wide range of silencing pathways including RNAi, co-suppression (PTGS), and quelling is now collectively called RNA silencing and appears to be present in most, if not all, eukaryotic organisms. The common key player in RNA silencing is small RNA of 21-28 nucleotides (nt) in length. Two classes of small RNAs are involved in RNA silencing: small interfering RNAs (siRNAs) and microRNAs (miRNAs).

Because of the exquisite specificity and efficiency, RNAi has drawn much attention as a powerful gene knockdown technique. Previous knockdown techniques, such as antisense oligonucleotides and ribozymes, usually show low efficiency in vivo and require empirical screening of a number of candidates before acquiring effective molecules (6). SiRNAs used for RNAi can inhibit gene expression over 90% in most genes. Although a guideline for constructing the best siRNA is not

established yet, testing 3-4 candidates are usually sufficient to find effective molecules. Technical expertise accumulated in the field of antisense oligonucleotide and ribozyme is now being quickly applied to RNAi, rapidly improving RNAi techniques.

In this review, the basic mechanism of small RNA-mediated silencing will be discussed. I will also describe current RNAi techniques and overview the current applications of RNAi in functional genomics and gene therapy.

SIRNA AND RNAI

RNAi is mediated by small interfering RNAs (siRNAs) that are generated from long dsRNAs of exogenous or endogenous origin (7-10). Long dsRNAs are cleaved by a ribonuclease III (RNase III) type protein Dicer. Dicer homologues can be found in *S. pombe*, *C. elegans*, *Drosophila*, plants, and mammals, suggesting that small RNA-mediated regulation is evolutionarily ancient and may have critical biological roles. SiRNA generated by Dicer is a short (~22-nt) RNA duplex with 2-nt overhang at each 3' end (Fig. 1). Each strand contains a 5' phosphate group and a 3' hydroxyl group. SiRNA is incorporated into a nuclease complex called RISC (RNA-induced silencing complex) that targets and cleaves mRNA that is complementary to the siRNA. The initial RISC containing a siRNA duplex is still inactive until it is transformed into an active form (RISC*) (11), which involves loss of one strand of the duplex by an RNA helicase activity. The iden-

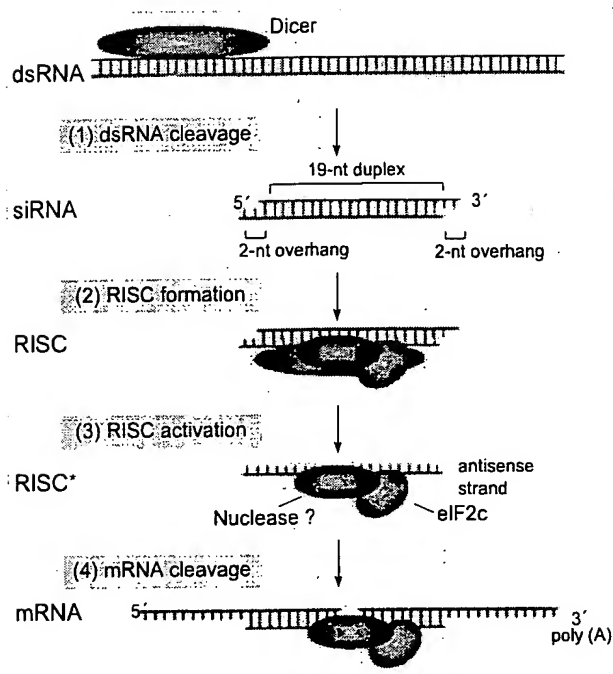


Fig. 1. Current model for RNA interference. RNAi process can be divided into four stages: (1) dsRNA cleavage by Dicer and generation of siRNA duplex, (2) recruitment of RNAi factors and formation of RISC (RNA-induced silencing complex), (3) siRNA unwinding and RISC activation, and (4) mRNA targeting and degradation.

tity of the RNA helicase is currently unknown. Dicer has a conserved helicase domain but it remains to be seen whether Dicer actually catalyzes this reaction.

Biochemical studies using *Drosophila* S2 cell extracts and human HeLa cell extracts revealed the presence of argonaute family proteins in the RISC. Argonaute-2 (AGO2) was found in *Drosophila* and two isoforms of eIF2C (eIF2C1 and eIF2C2) in human. Argonaute family proteins are ~100-kDa highly basic proteins that contain two common domains, PAZ and PIWI domains (12). PAZ domain consisting of ~130 amino acids is usually located at the center of the protein. The C-terminal PIWI domain containing ~300 amino acids is highly conserved. The functions of these domains are largely unknown but the PIWI domain of human eIF2C was recently shown to be essential for its interaction with Dicer (13). Depletion experiment of the eIF2C proteins by RNAi showed that they are required for RNAi (13). The biochemical functions of argonaute family proteins are still unclear.

The identity of the nuclease that executes the cleavage of mRNA remains elusive. Partially purified human RISC is estimated to be between 90 and 160-kDa leaving little room for an additional protein except for eIF2C (14). Genetic studies of *C. elegans*, *Drosophila*, *Neurospora crassa* and plants revealed several other genes that may be involved in RNA silencing although their biochemical roles remain to be determined.

Persistent RNAi has been observed in *C. elegans* (15) and

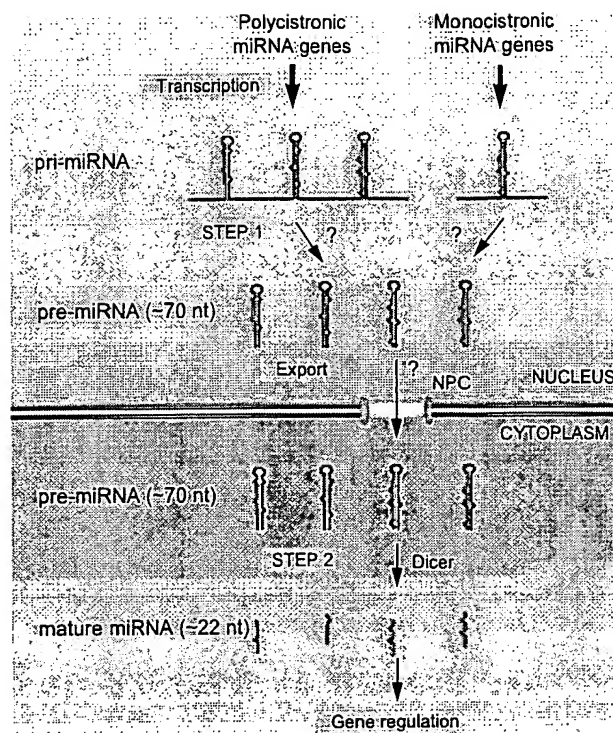


Fig. 2. A model for miRNA biogenesis and function. miRNA genes are transcribed by an unidentified polymerase to generate the primary transcripts, referred to as pri-miRNAs. Illustrated in the upper left is the clustered miRNA such as miR-23~27~24-2 of which the pri-miRNA is polycistronic. Illustrated in the upper right is the miRNA such as miR-30a of which the pri-miRNA is monocistronic. The first-step processing (STEP 1) releases pre-miRNAs of ~70-nt that is recognized and exported to the cytoplasm. The processing enzyme for the STEP 1 and the export factor are unidentified. Upon export, Dicer and possibly other factors participate in the second-step processing (STEP 2) to produce mature miRNAs. The final product may function in a variety of regulatory pathways, such as translational control of certain mRNAs. The question marks indicate unidentified factors.

N. crassa (16) but not in *D. melanogaster* (17) and mammals (18). RNAi in *C. elegans* can be transmitted to the progeny (F1) although the effect gradually diminishes. RNAi in human cells is transient and usually lasts less than five doubling times. It was reported that siRNAs are amplified by RNA-dependent RNA polymerase in nematode and fungi, while flies and mammals seem to lack this enzyme.

MICRORNA AND GENE SILENCING

Hundreds of small RNAs have been recently found in human as well as in *C. elegans*, *D. melanogaster*, and plants (19-29). These RNAs, termed microRNAs (miRNAs), are indistinguishable from active siRNAs in their biochemical properties. They are ~22-nt in length and contain 5' phosphate and

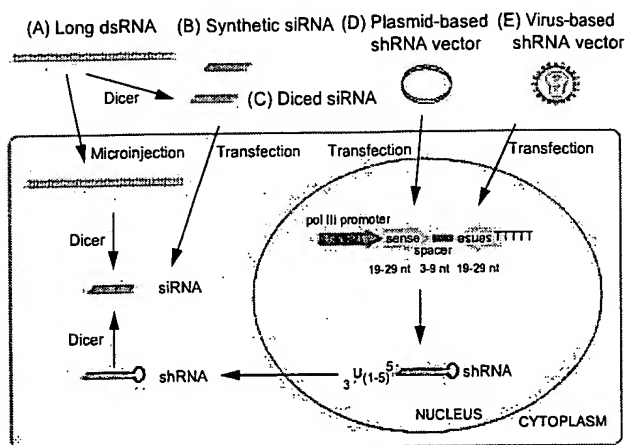


Fig. 3. Various strategies of RNAi in mammalian cells. (A) Long dsRNAs can induce specific RNAi in certain cell types such as oocytes and embryos. Injected dsRNA is converted to siRNA in the cell. (B) Chemically synthesized siRNA can be efficiently transfected into a variety of cells using lipophilic reagents. (C) siRNA can be prepared in vitro from dsRNAs using recombinant Dicer protein. Diced RNAs are purified based on their size (~21-nt) and transfected into cells. (D) Short hairpin RNAs (shRNAs) are expressed in the nucleus from expression plasmids. The pol III-derived expression system is shown here as an example. Upon export, shRNAs are processed by Dicer releasing siRNAs. (E) ShRNA expression cassette can be delivered by viral vectors such as retroviral vector, lentiviral vector, and adenoviral vector.

3' hydroxyl group. An interesting common feature of miRNAs, which is not shared by siRNAs, is that miRNA sequences are found in the stem of a stem-loop structure (19-22) (Fig. 2). The stem-loops are usually imperfect hairpins of over 70-nt with some bulges and internal loops. Recent studies showed that miRNAs are transcribed as long primary transcripts (pri-miRNAs) that are trimmed into the characteristic ~70-nt stem-loop forms (pre-miRNAs) (30) (Fig. 2). This initial processing occurs mainly in the nucleus (30). The identities of RNA polymerase and the nuclear processing enzyme have not been determined yet. Pre-miRNAs of ~70nt stem-loop get exported to the cytoplasm and subjected to the second processing to generate the final product of ~22-nt mature miRNAs. Dicer, the siRNA processing enzyme, executes the processing of ~70-nt RNAs into mature miRNAs (31-34).

The paradigm for the function of miRNAs has been originally provided by *lin-4* and *let-7* RNA, which were identified by genetic analysis of *C. elegans* developmental timing (35, 36). They were initially called small temporal RNAs (stRNAs) because of their temporal expression pattern and their roles in temporal regulation. *lin-4* and *let-7* RNA act as post-transcriptional repressors of their target genes when bound to their specific sites in the 3' untranslated region of the target mRNA (35, 37-40). The level of target mRNA does not change, suggesting that the inhibition occurs at the level of translation. Recently, *bantam* RNA from *Drosophila* has

been found to suppress apoptosis and stimulate cell proliferation by inhibiting translation of *hid* mRNA (41). *bantam* RNA is expressed in a temporal and tissue-specific manner, regulating tissue formation during development. Functions of hundreds of other animal miRNAs are currently unknown. However, given the diversity in sequences and expression patterns, miRNAs are expected to play various roles in a wide range of regulatory pathways (42).

Since animal miRNAs are only partially complementary to their target mRNAs (43), it is difficult to search for the target mRNA. Plant miRNAs are different from animal miRNAs in their action mechanism. Some plant miRNAs show high degree of complementarities to developmentally important mRNAs (44). Plant MIR39 and MIR165/166 were found to interact with specific mRNAs resulting in cleavage of the mRNAs, indicating that these miRNAs act like siRNAs (45, 46). Thus siRNA-mediated RNAi and miRNA-mediated translational inhibition appears to be determined only by the degree of complementarity between the small RNA and the target mRNA. Intriguingly, human *let-7* RNA was found to be a component of a RISC and can catalyze target cleavage if the mRNA has perfect complementarity to *let-7* RNA (47). Moreover, some components of miRNA-protein complex (miRNP) such as eIF2C2 overlap with those of RISC (14, 22, 47). It is an open question how RISC (RNAi machinery) and miRNP (miRNA-protein complex) are different in their compositions and functions.

PRACTICAL ASPECTS: TRANSFECTION OF SYNTHETIC siRNAS

While the discovery of RNAi revolutionized genetic studies in *C. elegans*, development of RNAi techniques in mammalian cells was belated because long dsRNA nonspecifically suppressed gene expression in mammalian cells. Long dsRNA (over 50 bp) activates dsRNA-dependent protein kinase (PKR) and 2', 5'-oligoadenylate synthetase leading to nonspecific translational inhibition and RNA degradation, respectively (48, 49). This pathway does not exist in embryonic stage, allowing specific RNAi in mouse oocytes and embryos (4, 50). RNAi in somatic cells was once thought to be not feasible but the limitation was soon circumvented by Thomas Tuschl's group and others by using synthetic siRNA duplexes (21-nt) that are too short to induce non-specific inhibition (51-53) (Fig. 3). This method involves transfection of synthetic siRNA into cultured cells. A detailed user guide for siRNA is given at Tuschl lab's homepage (www.rockefeller.edu/labheads/tuschl/sirna.html). Because of its straightforward protocol, siRNA transfection is the most widely used RNAi technique at present. Custom synthesis service for siRNA is available from Dharmacon RNA technologies, QIAGEN and Ambion. siRNA can be delivered to living organisms as well, which opens the possibility of applying RNAi in gene therapy. When siRNA was inject-

ed to the tail vein of postnatal mice, it was delivered to a variety of organs including the liver, kidney, spleen, lung, and pancreas (54, 55).

There are several factors that may influence the efficiency of RNAi in mammalian system (56). First, the choice of the target site is important. Originally, it was suggested that the best target site is around the first 100-nt downstream of the translation start site. However it is not clear yet which region on mRNA is most vulnerable to RNAi. Secondary structures and mRNA-binding proteins may influence the accessibility to siRNA, although no systematic study has been carried out to compare the entire region of a given gene. In practical terms, about four different siRNAs should be randomly chosen throughout the entire mRNA and experimentally tested to find the best working one. When designing siRNA, BLAST sequence analysis is needed to avoid a chance that the designed siRNA is complementary to other unrelated mRNA. Programs to design siRNA are now available at the Whitehead Institute's biocomputing home (jura.wi.mit.edu/bio) and the siRNA manufacturers' sites (www.dharmacon.com and www.qiagen.com).

Second, the transfection method makes a difference in the outcome. SiRNAs can be efficiently transfected into cells when lipophilic agents such as Oligofectamine™ and TransIt-TKO™ are used. Oligofectamine™ is most frequently used because of its low toxicity. Virtually 100% of HeLa and HEK293 cells can be transfected using this method. Primary cells and T cell lines that are usually difficult to transfect have also been transfected with siRNAs at relatively high efficiencies. Transfection conditions such as transfection reagent, cell density, and duration of incubation should be optimized for the given cell type and the targeted gene. Apart from transfection using lipophilic agents, electroporation have been successfully used for some cell types such as T cells (57) and human hepatoma cell line, Huh-7 (58). Massive cell death during electroporation (over 50%) should be taken into consideration depending on the particular application.

Third, the turnover rate of the protein should be taken into account because RNAi only aims at the mRNA not the protein itself. Generally, siRNA causes rapid reduction in mRNA levels in 18 hr or less but stable proteins require a longer period for depletion. SiRNA-mediated RNAi lasts only for 3-5 cell doubling times, probably due to gradual dilution of siRNA through cell division. Therefore multiple transfection is necessary in cases that the protein is unusually stable or the cells need to be grown for long time to observe the phenotype.

Fourth, it is possible that two or more highly expressed genes are knocked down simultaneously but two siRNAs seem to compete with each other, suggesting that the RNAi machinery (protein factors such as argonaute family proteins) may be limiting in human cells. Thus careful control of siRNA concentration is required for simultaneous knock-down of multiple genes.

Recently, an alternative method for siRNA preparation has

been developed. Long dsRNAs that are transcribed in vitro using T7 RNA polymerase (or other RNA polymerases) are incubated with recombinant Dicer to generate siRNAs (59, 60). The resulting diced products contain a mixture of siRNAs binding to multiple sites on a target mRNA, eliminating the need to design and test multiple individual siRNAs. Although this method requires longer hand-on time than synthetic siRNA method does, it is easier to design and less costly. SiRNA generation kits are available from several companies including Gene Therapy Systems, Inc.

PRACTICAL ASPECTS: DNA-BASED EXPRESSION OF SMALL HAIRPIN RNA (shRNA)

Despite of the potent knockdown capabilities, the siRNA transfection method has its weak points such as transient effect and difficulties in transfection depending on cell types. Stable gene silencing was achieved by developing a new method based on the expression of siRNAs from DNA templates (Fig. 3). The first type makes use of RNA polymerase III (pol III) promoter such as U6 promoter (61-66), H1 promoter (67-71) and tRNA^{val} promoter (72). An advantage of using the pol III system is that transcription terminates at a stretch of 4 thymidines, making it possible to produce short RNA with 1-4 uridines at the 3' end. When "short hairpin RNA" (shRNA) similar to miRNA is transcribed from pol III promoter, shRNA gets processed by Dicer to generate siRNAs. To construct an shRNA expression cassette, the gene-specific targeting sequence (19-29-nt sequences from the target transcript separated by a short spacer from the reverse complement sequences) is inserted between pol III promoter and terminator. The loop (spacer) sequences appear to affect silencing effect. For instance, it was suggested that two uridines placed at the base of the loop were important for efficient silencing (67). Kawasaki and Taira reported that the loop sequences from miRNA (miR-23) helped the nuclear export and processing of short hairpin, enhancing RNAi effect (72). Some argue that longer stem (up to 29nt) is more efficient than shorter ones (61, 73). However, there is no clear guideline to make the best hairpin at the moment. This is partly because we do not have sufficient understanding of small RNA processing. Therefore, studies on small RNA processing would be important for development of RNAi technique.

A similar approach to the shRNA method is to transcribe ~21-nt sense and antisense RNA separately from pol III promoters (64-66). Although this method appears to provide equally strong RNAi effects, it requires construction of two expression cassettes so it does not seem to be as practical as the shRNA strategy in most cases.

ShRNAs can also be generated from pol II promoters such as human cytomegalovirus (CMV) immediate early promoter (74). Using pol II promoter would be advantageous in terms of regulated expression of siRNA. A variety of inducible/

repressible promoters are available for specific expression. This type of shRNA expression system has not been widely used yet and needs further experiments to prove its efficiency.

The first-generation RNAi vectors were plasmid-based. A selectable marker was embedded in the same plasmid, allowing selection of transfected cells. However, plasmid-based shRNA expression has limitations in cases where transfection efficiency is low.

To overcome this problem, viral vectors were employed to deliver shRNA expression cassette. Retroviral vectors are most widely used among viral vectors for in vitro gene transfer and in vivo gene therapy. Murine retrovirus-based vectors were shown to be efficient in delivery of shRNA (68, 75-78). Lentivirus-based vectors were also tested and appear to be promising vehicles for RNAi because they are effective in infecting non-cycling cells, stem cells, zygotes and their differentiated progeny (70, 79). Adenoviral vectors are highly effective but allow only transient expression of siRNA (80, 81). This property may be advantageous in some applications such as cancer gene therapy, where persisted expression is not necessary (81).

FUNCTIONAL GENOMICS

In classical forward genetics, genes were first defined by the description of their phenotype, which is then followed by the search for each gene at the molecular level. With the whole human genome sequenced and the predicted ~30,000-40,000 protein-coding genes, reverse genetics to probe the function of the predicted genes seems more effective and reasonable strategy. However, reverse genetics using knockout technique by homologous recombination takes too much time and resources. Knockdown techniques such as antisense and ribozyme proved to be far less efficient in inhibiting gene expression for this purpose.

A breakthrough was made by discovery of RNAi in *C. elegans* in 1998 when Fire et al. reported that dsRNA can induce strong and specific silencing of homologous genes (1). RNAi can be induced in this nematode worm by injection of dsRNA into gonad, by soaking the worm in dsRNA or by simply feeding the worm of bacteria engineered to express dsRNA. RNAi is now being used for studies of individual genes as well as for genome-wide genetic screening. A bacterial library for inactivation of 16,757 of the worm's predicted 19,757 genes was developed and the corresponding phenotypes were listed (82, 114). The bacterial clones are reusable and have been used in screenings for genes with more specific functions such as body fat regulation, longevity, and genome stability (83-85).

Drosophila is another popular model organism where RNAi has been successfully used to study functions of individual genes (2, 86, 87). Unlike in *C. elegans* and plants, RNAi in *Drosophila* is not systematic, meaning that RNAi does not spread into other cells or tissues (17, 88). This property allows

cell-specific RNAi in *Drosophila*.

RNAi in cultured mammalian cells is quickly becoming a standard laboratory technique to study functions of individual genes. Transfection of synthetic siRNA has been most frequently used but other methods are quickly being developed as discussed above. Screening of human genome in a wider scale is also being considered. This would be much more painstaking than screening in *C. elegans* because number of human genes is about twice and RNAi technique in human is more complicated. However, efforts are already underway by developing libraries of siRNAs and automatic screening systems. Once the screening system is established, the long-standing goal of genome-wide functional genomics in human may become feasible, although the screening based on a cell line rather than the whole organism will have limitations.

RNAi may also be used to rapidly create transgenic mice. It was shown recently that retroviral or lentiviral delivery of shRNA-expression cassette can be passed through the mouse germline (79, 89). Thus, RNAi may complement standard knockout approaches and accelerate studies of gene function in living mammals.

MEDICAL APPLICATIONS

The idea of using RNAi for therapeutic purpose has been tested extensively for last two years (90) since Tuschl's pioneering work on siRNAs. Candidate diseases for such treatment include viral infection (91), cancer (92), and dominantly inherited genetic disorders.

Human immunodeficiency virus (HIV) was the first obvious target for such application. Viral genes including *tat*, *rev*, *nef*, and *gag* have been silenced, resulting in successful inhibition of viral replication in cultured cells (64, 93-99). Cellular genes such as CD4, CCR5 and CXCR4 that are necessary for viral infection were also targeted giving similar results (99, 100). Hepatitis C virus (HCV), a major cause of chronic liver disease, has a genome of a single-stranded RNA, making it an attractive target for RNAi. Expression of RNAs from HCV replicon was inhibited in cell culture, providing a hope for a new therapy for this virus (58, 101-104). Human papilloma virus (HPV) is believed to contribute to tumorigenesis. Silencing E6 and E7 genes of HPV type 16 by siRNA resulted in reduced cell growth and induced apoptosis in cervical carcinoma cells (105). Reduction in hepatitis B virus (HBV) RNAs and proteins has been induced by siRNA-producing vectors in cell culture (106) and in mouse liver (107). Influenza virus was also challenged with siRNA specific for nucleocapsid (NP) or a component of the RNA transcriptase (PA), which abolished the accumulation of viral mRNAs (108). These successful results are encouraging but there would be many hurdles to achieve viral eradication in vivo. Further experiments are needed in animal models as well as in clinical settings.

Exquisite sequence specificity of RNAi enables specific knockdown of mutated genes. Such possibility was first tested on an oncogene, *K-RAS* (V12), whose loss of expression led to loss of anchorage-independent growth and tumorigenicity (76). Employing a retroviral version of the H1 promoter-driven shRNA expression system (67), the authors showed strong inhibition of the expression of mutated *K-RAS* (V12) while leaving other ras isoforms unaffected. This approach was particularly encouraging because it was successful not only in tissue culture but also in an animal model (mouse). Similar studies quickly followed using various forms of siRNA. Oncogenes can be activated by chromosomal translocation fusing two parts of unrelated genes. M-BCR/ABL fusion leads to leukemic cells with such a rearrangement. Transfection of dsRNA specific for the *M-BCR/ABL* mRNA has been shown to downregulate the fusion protein in K562 cells (109). Overexpression of oncogenes is another cause of tumorigenesis. Overexpression of P-glycoprotein (*P-gp*), the MDR1 gene product, confers multidrug resistance (MDR) to cancer cells. RNAi successfully reduced P-gp expression and thereby drug resistance (110). Expression of endogenous erbB1 can be suppressed by RNAi in A431 human epidermoid carcinoma cells (111). Combined RNAi to reduce expression of *c-raf* and *bcl-2* genes may also represent a novel approach to leukemia (112). Blocking angiogenesis is another important anti-cancer strategy. Vascular endothelial growth factor (VEGF) exists as at least five isoforms that are thought to perform different functions in tumor angiogenesis. Specific knock-down is possible by using RNAi, providing a new tool to study isoform-specific VEGF function as well as to treat cancer (71).

Dominantly inherited genetic disorders are usually caused by mutations on one allele whose gene product acts transdominantly. Specific abrogation of the mutated gene would leave the unaffected allele to restore the normal cellular function. Expansion of trinucleotide (CAG) repeats encoding an increased polyglutamine tract causes at least eight human neurodegenerative disorders, including Huntington's disease and spinobulbar muscular atrophy (Kennedy's disease). Although the mechanism underlying neurodegeneration is unclear, aggregation of mutant polyglutamine proteins is related to the toxic gain-of-function phenotype. SiRNA targeting the 5'-end or 3'-end of the CAG repeat rescued the polyglutamine toxicity in cultured cells (80, 113), opening the possibility for new approaches.

Other diseases considered for RNAi-based therapy include Fas-induced fulminant hepatitis (55). Intravenous injection of siRNA targeting *Fas* reduced Fas expression in mouse hepatocytes leading to resistance to apoptosis and protection of mice from liver fibrosis.

There are several critical hurdles to be circumvented before RNAi becomes a realistic tool in clinics. First, enough amount of siRNA should be delivered into enough number of target cells, efficiently and stably. This delivery problem may be solved by chemically modifying siRNA to make it more sta-

ble, penetrable, and cost-effective. Alternatively, siRNAs can be delivered by way of viral vectors. Viral vectors such as lentiviral vectors would have unique advantages over synthetic siRNAs in terms of persistency. Developing optimal vectors will greatly accelerate siRNA-mediated gene therapy. A related issue is "targeted" administration of siRNA. This is hard to be achieved with synthetic siRNAs. For DNA-based RNAi, however, inducible/repressible promoters can be used to regulate siRNA expression in a tissue specific or developmental stage specific manner. The second problem stems from the technique's own merit; sequence specificity. Frequent mutations of target genes may allow escape from specific inhibition of disease genes, especially in viral infection. A "combination" strategy using several different siRNAs is likely to minimize the escape.

PERSPECTIVES

The recent discovery of small RNAs is fascinating. For decades we have been ignoring these tiny molecules as mere degradation products of bigger transcripts. Mutations in intergenic regions were often dismissed during genetic screening. Genomics has focused on protein-coding genes leaving non-coding RNA genes unnoticed. Now hundreds of small RNA genes have been discovered. Understanding their biology is likely to reveal novel aspects of cellular functions. Key pressing questions include what their functions are and how these unusual RNA genes are expressed.

Technical aspects of small RNA biology are also important. RNAi is already changing our way of studying gene functions. However, not every promising technique is translated into commercial or clinical success. RNAi, too, should go through intensive elaboration and further innovation. Setting a guideline for selection of siRNA sequence would be an important step. Efficient delivery and regulated expression of siRNA are also critical issues for transgenic studies and gene therapy. In addition, developing a simple and inexpensive RNAi protocol for high throughput screening will be essential for large-scale genomics.

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